

The *Mycobacterium tuberculosis* protein Ldt_{Mt2} is a nonclassical transpeptidase required for virulence and resistance to amoxicillin

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The peptidoglycan layer is a vital component of the bacterial cell wall. The existing paradigm describes the peptidoglycan network as a static structure that is cross-linked predominantly by 4→3 transpeptide linkages. However, the nonclassical 3→3 linkages predominate the transpeptide networking of the peptidoglycan layer of nonreplicating *Mycobacterium tuberculosis*^{1,2}. The molecular basis of these linkages and their role in the physiology of the peptidoglycan layer, virulence and susceptibility of *M. tuberculosis* to drugs remain undefined. Here we identify MT2594 as an L,D-transpeptidase that generates 3→3 linkages in *M. tuberculosis*. We show that the loss of this protein leads to altered colony morphology, loss of virulence and increased susceptibility to amoxicillin-clavulanate during the chronic phase of infection. This suggests that 3→3 cross-linking is vital to the physiology of the peptidoglycan layer. Although a functional homolog exists, expression of *ldt*_{Mt2} is dominant throughout the growth phases of *M. tuberculosis*. 4→3 transpeptide linkages are targeted by one of the most widely used classes of antibacterial drugs in human clinical use today, β-lactams. Recently, meropenem-clavulanate was shown to be effective against drug-resistant *M. tuberculosis*³. Our study suggests that a combination of L,D-transpeptidase and β-lactamase inhibitors could effectively target persisting bacilli during the chronic phase of tuberculosis.

Tuberculosis continues to be a major public health threat around the world. The estimate that more lives may have been lost in 2009 due to tuberculosis than in any year in history is alarming⁴. An increasing number of cases reporting infection with multidrug-resistant and extensively drug-resistant (XDR) strains of *M. tuberculosis* have diminished the capability to respond effectively against this threat. A recent study reporting high mortality rates of individuals concurrently infected with HIV and XDR tuberculosis illustrates the need for new drugs to treat tuberculosis⁵. A major reason for the emergence of drug resistance is thought to be poor compliance to treatment regimens, as the current therapy requires a combination of drugs to be taken daily for 6 months or more⁴. Although >99% of *M. tuberculosis* bacilli are killed

within 2 weeks, it takes the remainder of the therapy to effectively kill the surviving population⁶. These bacilli, broadly defined as ‘persisters’, are able to transiently tolerate drugs. The phenomenon of persistence is poorly understood. *In vitro* models designed to mimic the physiology of persisters are based on exposure to nitric oxide and depletion of oxygen and nutrients, as these conditions are thought to prevail in a persisting infection *in vivo*^{7–9}.

A higher percentage of *Escherichia coli* bacilli are able to survive exposure to drugs and persist at stationary phase as compared to bacteria in the exponential phase of growth¹⁰. Little is known about changes in the cell wall during the chronic phase of infection and whether it regulates persistence of *M. tuberculosis* in the host. Understanding the regulation of cell wall physiology and its consequences may enable us to effectively target and kill persisters by interfering with this process. The cell wall of *M. tuberculosis* accounts for up to 40% of the cell’s dry mass, as compared to 5% and 10% in Gram-positive and Gram-negative bacteria, respectively¹¹. It is thought that the degree of peptidoglycan cross-linking is ~50% in *E. coli*¹², whereas it is ~70%–80% in *Mycobacterium* species¹³. Mycobacterial peptidoglycan layer is cross-linked with both 4→3 and 3→3 linkages¹. Recently, it was shown that 80% of the transpeptide linkages in the peptidoglycan of *M. tuberculosis* at stationary phase are of the nonclassical 3→3 type². Here we report identification of a gene, MT2594 (hereafter reannotated as *ldt*_{Mt2}), that encodes an L,D-transpeptidase for synthesis of nonclassical 3→3 cross-linkages and show that inactivation of the gene results in altered colony morphology, attenuation of persistence and increased susceptibility to amoxicillin/clavulanate both *in vitro* and in the mouse model of tuberculosis.

We isolated a mutant *M. tuberculosis* resulting from inactivation of *ldt*_{Mt2}, by screening a collection of 5,100 unique transposon insertion mutants for growth attenuation¹⁴. Colonies of this mutant (*ldt*_{Mt2}::Tn) were small, smooth and had punctuated aerial growth rather than the typical large, rough and laterally diffuse morphology observed in the parent strain (Fig. 1a), despite the lack of difference in the composition of the lipids that constitute the cell wall (Supplementary Fig. 1). Liquid broth cultures of the mutant strain reached lower optical and cell densities compared with the parental strain (Fig. 1b). The wild-type

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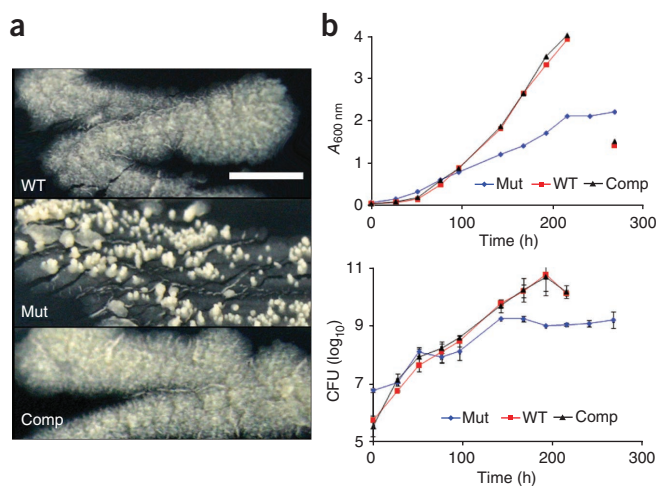


Figure 1 Morphology and growth *in vitro*. (a) Morphologies of wild-type *M. tuberculosis* (WT), Ldt_{Mt2} -mutant *M. tuberculosis* (Mut) and the complemented strain (Comp) on solid medium after 21 d of growth at 37 °C. Scale bar, 1 cm. (b) Growth of wild-type *M. tuberculosis*, Ldt_{Mt2} -mutant *M. tuberculosis* and the complemented strain in Middlebrook 7H9 liquid medium at 37 °C. The decrease in absorbance (top) and CFUs (bottom) at the final time point for the wild-type and complemented strains is due to clumped cultures.

phenotype was restored upon complementation of the mutant with a single copy of the gene (Fig. 1a,b). The ratio of colony-forming units (CFUs) of the mutant to WT at the beginning of the growth assay was 10:1, whereas after 192 h it was 1:100. These data show that there was a ~1,000-fold larger increase in the wild-type population over 192 h compared to the mutant strain. We derived doubling times of 18.1 h, 14.5 h and 14.8 h from CFU data for the mutant, wild-type and complemented strains, respectively.

Ldt_{Mt2} is annotated as a hypothetical protein with an unknown function. The amino terminus contains a putative single transmembrane

domain (positions 20–42), anchoring the remainder of the protein. The carboxy-terminal region of MT2594 is similar to the catalytic domain of the prototypic peptidoglycan L,D-transpeptidase from *Enterococcus faecium* (29% identity), including the active site cysteine residue within the invariant SHGC motif¹⁵ (Fig. 2a). To determine whether ldt_{Mt2} encodes a functional L,D-transpeptidase, we produced a soluble fragment of the protein in *E. coli* that we purified and assayed for cross-linking activity (Fig. 2b). The substrate was a disaccharide-tetrapeptide monomer isolated from the peptidoglycan of *Corynebacterium jeikeium*, which has the same structure as the predominant monomer of *M. tuberculosis*¹⁶. Electrospray mass spectrometry analysis of the reaction products revealed the formation of a peptidoglycan dimer ($m/z = 1,786.87 [M + H]^+$, Fig. 2c) from two disaccharide-tetrapeptide monomers ($m/z = 938.44 [M + H]^+$). Tandem mass spectrometry of the dimer confirmed the presence of a 3→3 cross-link connecting two mesodiaminopimelic acids at the third position of the stem peptides (Fig. 2c). Ldt_{Mt2} did not catalyze the formation of dimers with disaccharide-peptide containing a stem pentapeptide. Thus, Ldt_{Mt2} is specific for stem tetrapeptides, as are Ldt_{Mt1} (ref. 2) and the prototypic L,D-transpeptidase from *E. faecium*¹⁵. These results show that MT2594 is an L,D-transpeptidase that catalyzes the formation of 3→3 peptidoglycan cross-links.

Further investigation revealed four putative paralogs of ldt_{Mt2} in the genome of *M. tuberculosis* (MT0125, MT0202, MT0501 and MT1477). To gain an insight into the level of expression of the five paralogs, we performed quantitative RT-PCR analyses on eight RNA samples prepared from the exponential and stationary phases of growth. The ldt_{Mt2} messenger RNA was at least fivefold more abundant than the combined expression of the four paralogs (Supplementary Fig. 2). Next, we assessed their functional relevance to L,D-transpeptidation. Mutants lacking MT0202, MT0501 and MT1477 have morphologies and growth phenotypes similar to those of the parent wild-type strain (Supplementary Fig. 3). We purified MT0202, MT0501 and MT1477 but did not detect any L,D-transpeptidase activity with the peptidoglycan precursor as the substrate (data not shown).

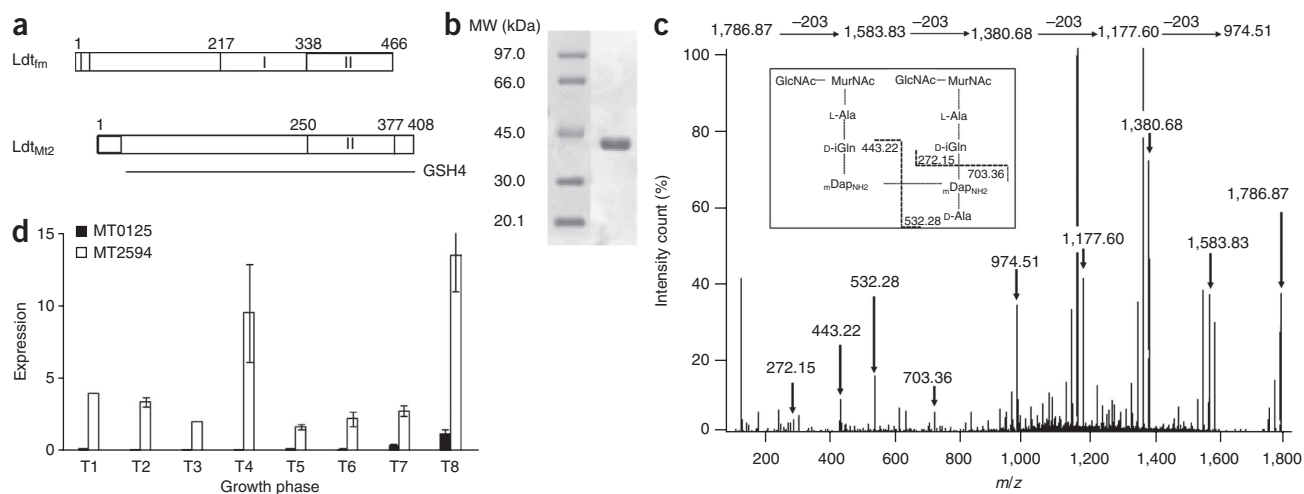


Figure 2 Characterization of Ldt_{Mt2} from *M. tuberculosis*. (a) Domain composition of L,D-transpeptidases from *E. faecium* (Ldt_{fm}) and *M. tuberculosis* (Ldt_{Mt2}). Residues 250–377 of Ldt_{Mt2} share homology with the catalytic domain of Ldt_{fm} (domain II, 338–466). (b) Purification of a soluble fragment of Ldt_{Mt2} produced in *E. coli*. (c) Structure and inferred fragmentation pattern of the peptidoglycan dimer formed *in vitro* by Ldt_{Mt2} . The ion at m/z 974.51 was generated by losses of the two *N*-acetylglucosamine-*N*-acetylmuramic acid (GlcNAc-MurNAc) residues after fragmentations of the ether links connecting the lactoyl group to the C-3 position of MurNAc. Loss of each sugar decreased the m/z by 203. Cleavage of additional peptide bonds from the ion at m/z 974.51 gave ions at 703.36, 532.28, 443.22 and 272.15, as indicated in the inset. (d) Transcription profile of Ldt_{Mt2} (MT2594) and Ldt_{Mt1} (MT0125). RNA isolated from wild-type *M. tuberculosis* cultures at growth phases denoted by time points T1 ($A_{600nm} = 0.2$), T2 ($A_{600nm} = 0.5$), T3 ($A_{600nm} = 0.8$), T4 ($A_{600nm} = 0.9$), T5 ($A_{600nm} = 0.19$), T6 ($A_{600nm} = 3.0$), T7 (2 d after $A_{600nm} = 3.0$) and T8 (3 d after clumping).

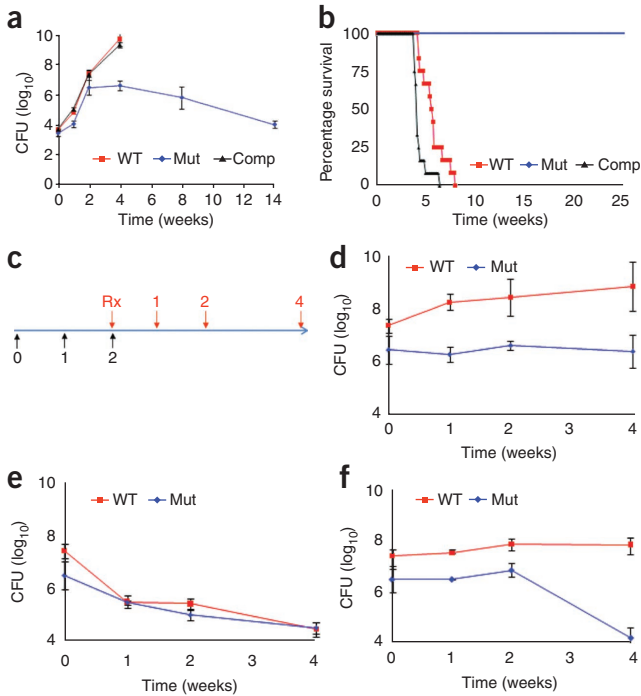


Figure 3 Assessment of growth, virulence and susceptibility to amoxicillin *in vivo*. (a) Bacterial burden in the lungs of mice infected with wild-type *M. tuberculosis*, *Ldt*_{Mt2}-mutant *M. tuberculosis* or the complemented strain. (b) Survival of mice infected with the three strains of *Mtb*. (c–f) Mice were infected with either wild-type or *Ldt*_{Mt2}-mutant *M. tuberculosis*. After 2 weeks of infection, mice were treated daily for 4 weeks (c), with either no drug placebo (d), 25 mg per kg body weight isoniazid (e) or 200 mg per kg body weight amoxicillin and 50 mg per kg body weight clavulanate (f). Bacterial burden was determined by enumerating CFU from the lungs of mice.

MT0125 of *M. tuberculosis* strain CDC1551 is identical to *Rv0116c* of strain H₃₇Rv, a gene designated *ldt*_{Mt1} that was recently shown to also encode a peptidoglycan L,D-transpeptidase². Of the two L,D-transpeptidase-encoding genes, *ldt*_{Mt2} was expressed at a level at least tenfold higher than *ldt*_{Mt1} at all phases of growth (Fig. 2d). The morphology, growth and virulence-deficient phenotype of the *ldt*_{Mt2} mutant indicates that low-level expression of *ldt*_{Mt1} does not compensate for the loss of the L,D-transpeptidase activity of *MT2594*, although analysis of the peptidoglycan structure showed that 3→3 cross-linkages were synthesized by *Ldt*_{Mt1} in stationary phase culture of the mutant (Supplementary Fig. 4).

Next, we assessed whether inactivation of *ldt*_{Mt2} affects the *in vivo* growth and virulence of *M. tuberculosis*. We implanted approximately 3×10^3 CFUs of each strain in the lungs of three groups of immunocompetent BALB/c mice. The *ldt*_{Mt2} mutant established infection and showed a normal growth pattern during the first 2 weeks but stopped proliferating beyond this stage of infection (Fig. 3a and Supplementary Fig. 5). The wild-type and the complemented strains proliferated rapidly until 4 weeks after infection, at which stage a heavy bacterial burden

caused the mice to die (Fig. 3a). The median survival times for the wild-type-infected and complemented strain-infected groups were 38 d and 30 d, respectively (Fig. 3b). The mice infected with the mutant strain did not die, and we did not observe any signs of morbidity despite the presence of $\sim 1 \times 10^4$ CFUs in the lungs during the persistent stage of infection (Fig. 3b).

The nonclassical 3→3 linkages comprises the majority of the transpeptide linkages in nonreplicating *M. tuberculosis*². We hypothesized that the loss of *Ldt*_{Mt2} may compromise the mutant's ability to adapt during the chronic phase of infection, a crucial stage in the pathogenesis of tuberculosis. If the failure in adaptation was the result of a defect in peptidoglycan cross-linking by *Ldt*_{Mt2}, another consequence could be an increased susceptibility to β-lactams. We tested this hypothesis by assessing the susceptibility of the *Ldt*_{Mt2} mutant to amoxicillin. *M. tuberculosis* produces a β-lactamase that is inactivated by clavulanic acid¹⁷. The minimum inhibitory concentration (MIC) of the commercially available clavulanic acid–amoxicillin combination (Augmentin) was 1.2 μg ml⁻¹ and 0.14 μg ml⁻¹ for the wild-type and mutant strains, respectively. Loss of *MT2594* did not alter susceptibility to isoniazid (MIC = 0.03 μg ml⁻¹) and D-cycloserine (5 μg ml⁻¹). Thus, loss of *Ldt*_{Mt2} was associated with increased susceptibility to amoxicillin in the presence of the clavulanic acid that inhibited the β-lactamase. A recent study assessed the susceptibility of the drug-sensitive laboratory strain H₃₇Rv and 13 XDR strains of *M. tuberculosis* to amoxicillin³. Whereas H₃₇Rv was found to be resistant to amoxicillin-clavulanate, all 13 XDR strains were highly susceptible, with an MIC ranging from 0.32 μg ml⁻¹ to 10 μg ml⁻¹. It has been reported that amoxicillin-clavulanate lacks early bactericidal activity, a measure of effectiveness of the drugs during the first 2 d of treatment¹⁸. However, amoxicillin-clavulanate has also been used to treat patients with multidrug-resistant tuberculosis¹⁹. An explanation for these observations is that amoxicillin-clavulanate lacks potency during the early phase but shows activity during the extended phase of treatment.

Next we assessed the susceptibility of the mutant strain to amoxicillin in mice. We treated mice infected with either the wild-type *M. tuberculosis* or the mutant strain with PBS as a placebo, 25 mg per kg body weight isoniazid or 200 mg per kg body weight amoxicillin plus 50 mg per kg body weight clavulanate (Fig. 3c–f). Isoniazid was similarly effective against both the wild-type and the mutant strains (Fig. 3e). The bacterial burden in mice infected with the wild-type strain treated with amoxicillin-clavulanate was similar to that in the group that received no treatment placebo (Fig. 3d,f). Amoxicillin-clavulanate

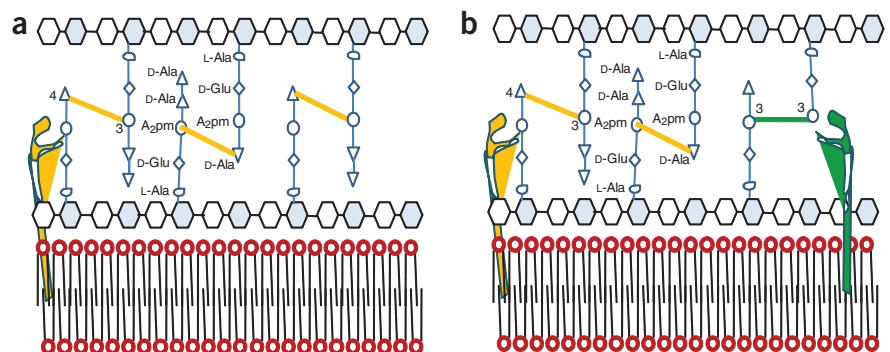


Figure 4 Proposed model for physiology of the peptidoglycan layer in *M. tuberculosis*. (a) A model of classical peptidoglycan cross-linking containing 4→3 interpeptide bonds. (b) A model based on our data: the peptidoglycan is cross-linked with classical 4→3 and nonclassical 3→3 interpeptide bonds. Both 4→3 (orange) and 3→3 (green) transpeptidases are involved in maintenance and remodeling of the peptidoglycan layer in the proposed model.

was ineffective during the first 2 weeks of treatment in mice infected with the mutant strain, as the bacterial burden remained unchanged (Fig. 3f). However, we observed a decrease in CFU of more than $2 \log_{10}$ between 2 and 4 weeks of treatment, illustrating that the mutant is susceptible to amoxicillin-clavulanate only during the chronic phase of infection (Fig. 3f).

All bacteria possess an elaborate peptidoglycan layer in their cell wall. In *E. coli*, the main interpeptide cross-linking occurs between the penultimate D-alanine at the fourth position of the donor and meso-diaminopimelate at the third position of the acceptor. Formation of this 4→3 bond is catalyzed by D,D-transpeptidases, which are the essential targets of β -lactam antibiotics²⁰. The drugs are structural analogs of the D-Ala-D-Ala extremity of the peptidoglycan precursors and act as suicide substrates in an acylation reaction²¹. *E. coli* has served as the model organism for studying peptidoglycan metabolism, but the existing paradigm built upon this organism is incomplete. In this classical model, the peptidoglycan layer is regarded as a static network involving polymerization of glycan chains and cross-linking of adjacent chains by formation of 4→3 peptide bonds (Fig. 4a)²². On the basis of our current data and our recent findings², we propose a model representing the peptidoglycan layer as a dynamic structure whose interpeptide linkages are altered as an adaptive response to a change in the environment and growth phase (Fig. 4b). The peptide chains of the peptidoglycan layer are linked with both 4→3 and 3→3 bonds.

In addition to transpeptidases, endo- and carboxypeptidases are also present in bacteria and function to modify the peptidoglycan network²³. These enzymes have yet to be definitively identified in *M. tuberculosis*. A recent report showed that the gene *pgdA* encodes an N-deacetylase that is involved in modification of the peptidoglycan layer in *Listeria monocytogenes*²⁴. A putative homolog of *pgdA* exists in *M. tuberculosis*. This gene, *MT1128* (Rv1096), has yet to be characterized, and its *in vivo* function has not been identified. Although MT2594 is a 3→3 transpeptidase, its loss and the accompanying changes in cross-linking may have pleiotropic effects on the metabolism of the peptidoglycan layer.

In this report, we have shown a new molecular basis of 3→3 linkages and their physiological role for viability and virulence of *M. tuberculosis*. We have also shown that L,D-transpeptidation is required for resistance to killing by amoxicillin-clavulanate and that inhibition of Ldt_{Mt2} alone may be sufficient to target the 3→3 linkages, despite the presence of Ldt_{Mt1}. It may be inferred from our findings that both 3→3 and 4→3 transpeptide linkages need to be destroyed to effectively kill *M. tuberculosis*. We have presented an unexploited enzyme in the pathway that can be targeted by one of the most successful groups of antibiotics in human clinical use, β -lactams. Therefore, a regimen containing a combination of inhibitors of L,D-transpeptidase and β -lactamase and a β -lactam may be able to kill *M. tuberculosis* by comprehensively destroying the peptidoglycan layer. As the peptidoglycan layer is a vital structure of the bacterial cell wall, insight and applications resulting from studies in *M. tuberculosis* will probably be broadly applicable to other bacteria.

METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/naturemedicine/>.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

R.G., W.R.B. and G.L. designed the project. J.-L.M. and M.A. designed the biochemical characterization of MT2594. M.L. and J.-L.M. performed biochemistry and analyzed data. R.G. and G.L. conducted genetics, microbiology and mouse experiments. G.L. wrote the manuscript with contributions from the other authors.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Bacterial strains and culture conditions. We used *M. tuberculosis* CDC1551, a clinical isolate, as the host strain to generate a transposon insertion mutant in *MT2594* (*ldt_{Mt2}::Tn*), as previously described¹⁴. This mutant carries a Himar1 transposon insertion at 872 bases downstream from the putative translation start site of the gene. Next, we generated a complemented strain by transforming the mutant with pGS202_2594. This is a single-copy integrating plasmid based on the pMH94 backbone²⁵, which we modified into a GATEWAY-compatible (Invitrogen) destination vector (Invitrogen). We cloned a wild-type copy of *MT2594* along with its promoter into the destination vector pGS202 to generate pGS202_2594. We verified genotypes of the strains by Southern blotting. We used Middlebrook 7H9 liquid medium supplemented with 0.2% glycerol, 0.05% Tween-80, 10% vol/vol oleic acid–albumin–dextrose–catalase and 50 µg ml⁻¹ cycloheximide for *in vitro* growth and Middlebrook 7H11 solid medium (Becton-Dickinson) for enumerating CFUs in *in vitro* and *in vivo* growth studies.

Production and purification of recombinant Ldt_{Mt2}. We amplified a portion of *ldt_{Mt2}* with primers 5'-TTTTCATGATCGCCGATCTGCTGGTGC-3' and 5'-TTGGATCCCGCCTTGCGCTTACCGGC-3' digested with BspHI-BamHI and cloned into pET2818¹⁵. The resulting plasmid encodes a fusion protein consisting of a methionine specified by the ATG initiation codon of pET2818, residues 55 to 408 of Ldt_{Mt2} and a C-terminal polyhistidine tag with the sequence GSH₆. We grew *E. coli* BL21 (DE3) harboring pREP4GroESL²⁶ and pET2818Ldt_{Mt2} at 37 °C in 3 l of brain heart infusion broth containing 150 µg ml⁻¹ ampicillin and induced expression with isopropyl-D-thiogalactopyranoside. We purified Ldt_{Mt2} from a clarified lysate by affinity chromatography on Ni²⁺-nitrilotriacetate-agarose resin (Qiagen) followed by anion exchange chromatography (MonoQ HR5/5, Amersham Pharmacia) with a NaCl gradient in 50 mM Tris-HCl pH 8.5. We performed an additional size-exclusion chromatography on a Superdex HR10/30 column equilibrated with 50 mM Tris-HCl (pH 7.5) containing 300 mM NaCl. Finally, we concentrated the protein by ultrafiltration (Amicon Ultra-4 centrifugal filter devices, Millipore) and stored it at -20 °C in the same buffer supplemented with 20% glycerol.

L,D-transpeptidase assays. We purified disaccharide-tetrapeptide containing amidated *meso*-diaminopimelic acid (GlcNAc-MurNAc-L-Ala¹-D-iGln²-*meso*-D-apNH₂³-D-Ala⁴) from *C. jeikeium* strain CIP103337 and determined the concentration after acid hydrolysis^{27,28}. Next, we tested *in vitro* formation of muropeptide dimers in 10 µl of 50 mM Tris-HCl (pH 7.5) containing 300 mM NaCl, 5 µM Ldt_{Mt2} and 280 µM disaccharide-tetrapeptide. We incubated the reaction mixture for 2 h at 37 °C and analyzed the resulting muropeptides by nano-electrospray tandem mass spectrometry, using N₂ as the collision gas²⁸.

Growth and virulence analysis in mice. We used 4- to 5-week-old female BALB/c mice (Charles River Laboratories) to study the *in vivo* virulence of the strains and their susceptibility to drugs. We infected mice with a log-phase culture of wild-type *M. tuberculosis*, *ldt_{Mt2}::Tn* *M. tuberculosis* or the

complemented strain in an aerosol chamber. For assessing *in vivo* growth of each strain, we killed 4 mice per group at days 1, 7, 14, 28, 56 and 98 after infection, obtained lungs and spleen, and homogenized and cultured appropriate dilutions on Middlebrook 7H11 medium to determine CFU. We allocated 12 mice for each infection group to assess the virulence of each strain, for which we determined the median survival time that mice from each group survived after infection. Protocols for experiments involving mice were approved by the Johns Hopkins University Animal Care and Use Committee.

Drug susceptibility testing in mice. We determined the minimum inhibitory concentrations for amoxicillin-clavulanate, imipenem (Merck), isoniazid (Sigma) and cycloserine (Sigma) by the broth dilution method²⁹. We used Augmentin (GlaxoSmithKline), a preparation containing amoxicillin and clavulanate as *M. tuberculosis* contains β-lactamases¹⁷. For this we inoculated 1 × 10⁵ *M. tuberculosis* bacilli in 2.5 ml of 7H9 broth and added drugs at concentrations ranging from 0.05–11.1 µg ml⁻¹. We incubated these cultures at 37 °C and evaluated them for growth by visual inspection at 7 and 14 d. For those samples with diminished growth compared to no-drug control, we determined CFUs. We used 4- to 5-week-old, female BALB/c mice for *in vivo* assessment of susceptibility of *M. tuberculosis* lacking Ldt_{Mt2} to amoxicillin. We infected two groups of mice, 36 per group, with aerosolized cultures of either wild-type *M. tuberculosis* or *ldt_{Mt2}::Tn*. We allocated 12 mice from each group into three subgroups and initiated daily treatment at 2 weeks after infection. We provided each subgroup with either 25 mg per kg body weight isoniazid, 200 mg per kg body weight amoxicillin and 50 mg per kg body weight clavulanate or no drug at all by oral gavage. For analysis, we killed 4 mice from each treatment subgroup at 1, 2 and 4 weeks after initiation of therapy, obtained lungs, homogenized them in 1 ml of PBS and determined CFUs in each organ by plating appropriate dilutions of the homogenates on Middlebrook 7H11 selective plates.

Additional methods. Detailed methodology is described in the **Supplementary Methods**.

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